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Purity, Cell Viability, Expression of GFAP and Bystin in Astrocytes Cultured by Different Procedures

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ABSTRACT

Primary astrocyte cultures are the most commonly used in vitro model for neurobiological studies. We speculated that different protocols might induce differences not only in the percentage of astrocytes but also in their biological characteristics. In this study, we investigated the effects of four major protocols on the purity of astrocytes, cell viability, expression of glial fibrillary acidic protein (GFAP) and bystin of cultured astrocytes using MTT assay, immunocytochemical staining, and Western blot analysis. We demonstrated that the purity of astrocytes (98.9%) generated by the subculture (SC) procedure is significantly higher than those generated by primary culture (PC), shaken once culture (SK-1) or shaken twice culture (SK-2). We also showed that expressions of GFAP and bystin in astrocytes that are purified by the SK-2 or SK-1 procedures are significantly higher than those in astrocytes prepared by PC or SC. In addition, astrocytes cultured by SK-2 or SK-1 have a higher level of cell viabilities at most time points after ischemia compared with astrocytes cultured by PC or SC. These suggested that physical stimulation induced by "shaken" or culture operation might be able to activate astrocytes and implied that different procedures induce differences not only in the purity but also in the biological characteristics of astrocytes, such as the percentage of activated astrocytes, GFAP, and bystin expressions and responses to ischemia. A more detailed analysis about the effect of "culture protocol factor" on the biological characteristics of astrocytes is absolutely needed. J. Cell. Biochem. 109: 30–37, 2010.

KEY WORDS: ASTROCYTES; PRIMARY CULTURE; SHAKEN ONCE CULTURE; SHAKEN TWICE CULTURE; SUBCULTURE; REACTIVE ASTROCYTES; ISCHEMIA/REPERFUSION; GLIAL FIBRILLARY ACIDIC PROTEIN (GFAP); BYSTIN

Astrocytes have been considered the supporting and housekeeping cells of the nervous system for a long time. Meanwhile, knowledge on the roles of the cells in the brain has dramatically extended in the last decade. Accumulated data show that the cells, instead of being a mere supporter of neurons, are the central integrators that govern all aspects of the birth, life and death

Zhong Ming Qian, Li Zhu, and Ya Ke contributed equally to this work.

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of neurons [Verkhratsky and Toescu, 2006]. It has been well documented that astrocytes are able to integrate neurons, synapses, and brain capillaries into individual and relatively independent units [Araque and Perea, 2004; Koehler et al., 2006; Verkhratsky and Toescu, 2006]. The astroglial syncytium connected through the gap junction communication pathways allows a rather elaborated intercellular communication route, which permits direct translocation of ions, metabolic factors, and second messengers [Pellerin, 2005; Abbott et al., 2006; Verkhratsky and Toescu, 2006]. These cells also play key roles in neuroinflammation [Darlington, 2005], repair [Sofroniew, 2005], and protection of neurons from pathological injury [Swanson et al., 2004]. Astrocytes influence neuronal survival by secreting nitric oxide, TNFalpha, matrix metalloproteinases, and other factors that can contribute to delayed neuronal death, and by producing erythropoietin to prevent programmed cell death after ischemic or excitotoxic stress [Swanson et al., 2004].

Much of the extended knowledge about the roles of astrocytes in the central nervous system (CNS) was obtained through in vitro studies using primary cultured astrocytes, the most commonly used in vitro model [Saura, 2007]. Since the original methods described by Booher and Sensenbrenner [1972] and McCarthy and de Vellis [1980], a great number of minor modifications have been incorporated into these protocols by different laboratories [Saura, 2007]. In our recent studies, we found that there were different responses to ischemia/reperfusion in astrocytes prepared by different procedures or protocols [Du et al., 2008]. This leads us to speculate that different protocols might induce differences not only in the percentage of astrocytes in the cultures but also in their biological characteristics. The latter might be the major reason for the differences in responses to ischemia/reperfusion. Currently, little is known about the effects of "culture protocol factor" on the biological characteristics of astrocytes. However, we believe that such information is essential for the precise interpretation of findings obtained from in vitro studies that use primary cultured astrocytes. This is also fundamental for a reasonable comparison of results among different laboratories that use different protocols in their primary astrocyte cultures. In this study, we therefore investigated the effects of four major protocols (primary culture, subculture (SC), shaken once culture and shaken twice culture) on the purity of astrocytes, cell viability, expressions of glial fibrillary acidic protein (GFAP) and bystin of cultured astrocytes. Our data showed that different protocols have significant effect not only on the purity but also on the biological characteristics of astrocytes. This effect should be not underestimated because it can significantly affect experimental results. A more detailed analysis about the effect of "culture protocol factor" on the biological characteristics of astrocytes is absolutely needed.

MATERIALS AND METHODS

MATERIALS

Unless otherwise stated, all chemicals were obtained from Sigma Chemical Company (St. Louis, MO). The astrocyte marker GFAP was purchased from Chemicon International Ltd (Millipore), (Temecula, CA) and the mouse anti-bystin antibody was a gift from Prof. Jiawei Zhou of Shanghai Institutes for Biological Sciences, China. Fetal bovine serum was obtained from Hyclone (Logan, UT) and Dulbecco's modified Eagle's medium from Gibco-BRL (Carlsbad, CA). The DC Protein Assay kit was bought from Bio-Rad Laboratories (Hercules, CA) and horseradish (HRP)-conjugated secondary antibodies from Pierce Chemical Company (USA).

PREPARATION OF MOUSE CEREBROCORTICAL ASTROCYTES

Cortical astrocytes were prepared from newborn ICR mice. Cortices were cut into small cubes ($<1\ mm^3$) and digested with 0.25% trypsin for 30 min at 37°C. Trypsinization was terminated by the addition of Dulbecco's modified Eagle's medium (Gibco) containing 10% fetal bovine serum, followed by mechanical trituration with a flame-polished Pasteur glass pipette. Cell suspensions were sieved through a 40 μm cell strainer. The filtrate was allowed pre-adherence for 30 min to remove any contamination from fibroblasts before being seeded at a density of 1×10^6 cells/cm^2 in 50 cm^2 flasks (Corning, USA) or in six-well plates (NUNC, Denmark). The plated cells were then treated with four different procedures. (1) *Primary culture (PC)*: The plated cells were cultured in a 5% CO_2 incubator (NAPCO 5400) at 37°C for 21 days and the culture medium was changed at 3-day intervals as described by Frangakis and Kimelberg [1984] and Tabernero et al. [1996]; (2) *Subculture (SC)*: The plated cells were incubated in a 5% CO_2 incubator at 37°C. After the cultures reached confluence (7 days), the cells were subcultured three times every 4 days. The cells were allowed pre-adherence for 30 min before being seeded in each SC process [Rzigalinski et al., 1997; Qian et al., 1999, 2000]. After the last SC, the cells were cultured for another 7 days before being used; (3) *Shaken once culture (SK-1)*: The plated cells were incubated in a 5% CO_2 incubator at 37°C. After the cultures reached confluence (7 days), the confluent cultures were shaken overnight on a rotary shaker (HZQ-C, HDL Apparatus, China) at 250 r/min for 12 h (37°C) as described by McCarthy and de Vellis [1980] and Gabryel et al. [2002]. (4) *Shaken twice culture (SK-2)*: The cells were treated as described in *SK-1* and the overnight shaking was repeated on the next day [Zwain and Yen, 1999; Innocenti et al., 2000]. The purity of astrocytes was assessed by the percentage of cells with GFAP fluorescence, accounting the total numbers of the nuclei stained by Hoechst 33342.

IMMUNOCYTOCHEMISTRY

Cells were first incubated with mouse anti-GFAP (1:1,000) for 24 h, followed by FITC-labeled goat anti-mouse IgG (1:100) (Sigma, USA, the secondary antibody). Hoechst 33342 (1 μg/ml) was then added to label all the nuclei. Fluorescent images were captured by using a fluorescence microscope (Leica Microsystems, Wetzlar, Germany) equipped with a SPOT cool CCD camera (S45, Canon, Japan) with a 490 nm excitation laser for GFAP' staining as green fluorescence and 350 nm excitation laser for Hoechst 33342 staining as blue fluorescence. The intensities of the fluorescence were measured and calculated by the value of the density of the green fluorescence to subtract the background using ImageJ software (from National Institutes of Health and available at http://rsb.info.nih.gov/ij/). Statistical analysis was performed using Student's *t*-test.

ASSESSMENT OF CELL VIABILITY

The cell viabilities of astrocytes that received different treatments were evaluated using an MTT (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyltetrazolinum bromide) assay as described by He et al. [2008]. Briefly, a total of 25 μl MTT (1 g/L in PBS) was added to each well and another 4 h of incubation at 37°C was conducted. The assay was stopped by the addition of a 100 μl lysis buffer (20% SDS in 50% *N′N*-dimethylformamide, pH 4.7). Optical density (OD) was measured at the 570 nm wavelength by the use of an ELX-800 microplate assay reader (Bio-tek, USA) and the results were expressed as a percentage of absorbance measured in the control cells.

WESTERN BLOT ANALYSIS

Astrocytes receiving different treatments were washed with ice-cold PBS and the proteins were extracted with 150 μl lysis buffer containing 50 mM Tris–HCl, 150 mM NaCl, 0.5% Igepal, 0.1% sodium dodecyl sulfate (SDS), 10 μg/ml phenylmethysulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 10 μg/ml of heat activated sodium ortovanadate. After centrifugation at 12,000*g* for 15 min at 4°C, the supernatant was collected. Protein content was determined using the DC Protein Assay kit (Bio-Rad Laboratories). A total of 5 μg protein was boiled in protein loading buffer for 5 min, separated on 10% SDS–polyacrylamide gels, and subsequently transferred to nitrocellulose membranes. Non-specific binding was inhibited by incubation in TBST [20 mM Tris-buffered saline (pH 7.5) with 0.1% Tween-20] containing 5% non-fat dried milk for 2 h at room temperature. The membranes were incubated with monoclonal antibodies against GFAP and bystin (1:10,000 in TBST containing 5% milk) overnight at 4°C. After three washes with TBST, the membranes were incubated for 2 h with horseradish (HRP)-conjugated secondary antibodies (Pierce Chemical Company) and developed using enhanced chemiluminescence (ECL Western blotting analysis system kit, Amersham Biosciences, England). The blot was detected using Kodak XAR-5 film for autoradiography. To ensure even loading of the samples, the same membrane was probed with anti-mouse β-actin monoclonal antibody (Sigma–Aldrich, MO) at a 1:10,000 dilution. The relative band density for bystin was calculated by dividing its band density by the density of β-actin as described in our previous study [Du et al., 2008].

STATISTICAL ANALYSIS

Statistical analyses were performed using SPSS 10.0. Data are presented as mean ± SEM. The difference between means was determined by one-way ANOVA followed by a Student–Newman–Keuls test for multiple comparisons. A probability value of $P < 0.05$ was taken to be statistically significant.

RESULTS

PURITIES OF ASTROCYTES CULTURED BY DIFFERENT PROCEDURES

Astrocytes were prepared from newborn ICR mice using different cultural procedures and their purity was assessed by the percentage of cells with GFAP fluorescence, accounting the total numbers of nucleus stained by Hoechst 33342 (Fig. 1). The purities of astrocytes obtained from PC, shaken once culture, shaken twice culture ,and SC are 88.9 ± 3.212%, 94.8 ± 1.325%, 96.5 ± 1.080%, and 98.9 ± 1.081%, respectively (Fig. 1A). The SC procedure generated astrocytes of the highest purity, which is significantly higher than those obtained from PC ($P < 0.01$), shaken once culture ($P < 0.01$), or shaken twice culture ($P < 0.05$). The purities of astrocytes cultured by the shaken once and shaken twice procedures are also significantly higher than those obtained from PC ($P < 0.05$ or 0.01).

Figure 1B-a–c showed the anti-GFAP immunocytochemical staining of astrocytes prepared by SC. Anti-GFAP immunocytochemical staining of astrocytes prepared by PC (Fig. 1B-d) and its corresponding Hoechst 33342 staining (Fig. 1B-e). The same nucleus was present in the image in Figure 1B-e stained by Hochest 33342, but there was an absence of GFAP immunocytochemical staining (Fig. 1B-d) in their cell bodies. This implied that there were many cells other than astrocytes in the culture. The same phenomenon also appeared in the image in Figure 1B-f (anti-GFAP immunocytochemical staining of astrocytes prepared by shaken twice culture) and Figure 1B-g (the corresponding Hoechst 33342 staining of Fig. 1B-f).

EXPRESSION OF GFAP PROTEIN IN ASTROCYTES CULTURED BY DIFFERENT PROCEDURES

Astrocytes were prepared from newborn ICR mice using different culture procedures with immunocytochemical staining performed afterwards. Observations under a fluorescence microscope showed that among the four groups, the staining of GFAP in the astrocytes obtained from the PC was the weakest (Fig. 2A-PC). Astrocytes purified by the shaken twice culture (SK-2) and shaken once culture (SK-1) procedures presented the strongest and the second strongest staining of GFAP among these groups, respectively, illminating in full-scale astrocytes except the nucleus (Fig. 2A-SK-2 and SK-1). The staining of subcultural astrocytes (Fig. 2A-SC) had the intermediate brightness among the groups. Fig. 2B showed data on the relative values of GFAP fluorescence density. The density in the SK-2 group was significantly higher than that in the PC or SC groups ($P < 0.05$). The density in the SK-1 group was significantly higher than that in the PC group ($P < 0.05$).

EXPRESSION OF BYSTIN PROTEIN IN ASTROCYTES CULTURED BY DIFFERENT PROCEDURES

It had been proposed that bystin might be a sensitive marker in activated astrocytes [Sheng et al., 2004; Du et al., 2008]. We therefore investigated the effects of different culture procedures on the expression of bystin protein in astrocytes. Western blotting analysis (Fig. 3) showed that shaken twice culture (SK-2) induced the highest expression of bystin protein, while shaken once culture (SK-1) induced the secondary highest. The bystin contents in these two groups were significantly higher than those in the astrocytes prepared by PC ($P < 0.001$) or SC ($P < 0.01$). The lowest expression of bystin was found in the astrocytes obtained from PC. Bystin content in this group was also significantly lower than that in the astrocytes prepared by SC ($P < 0.05$). The data suggest that physical stimulation induced by "shaking" or operation of SC might be able to activate astrocytes. Also, the findings imply that different culture procedures might induce differences not only in the purity of astrocytes but also in the percentage of activated astrocytes.

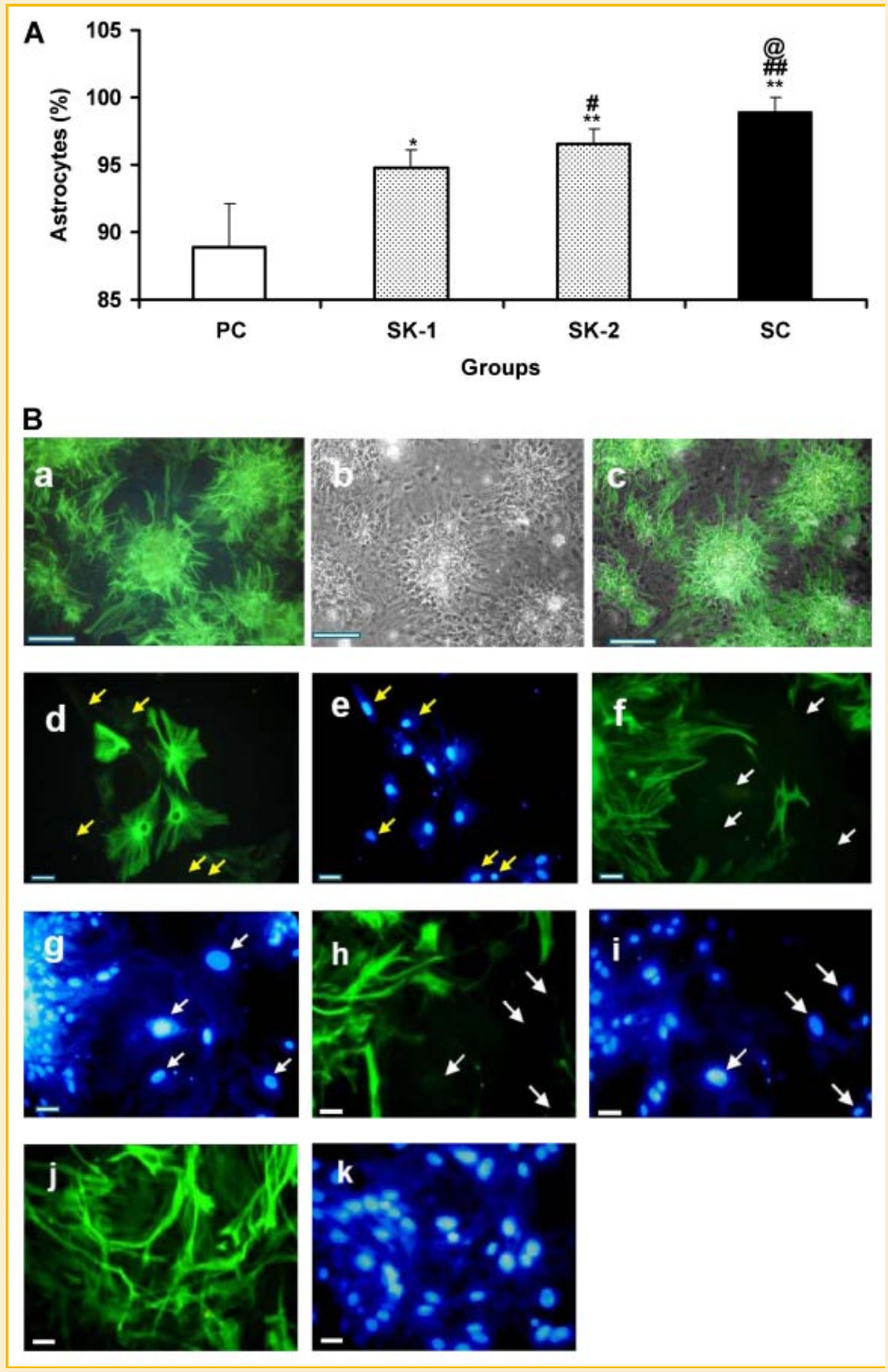

Fig. 1. The purities of astrocytes cultured by different procedures. Astrocytes were prepared from newborn ICR mice using different culture procedures, including primary culture, shaken once culture, shaken twice culture, and SC; the purities of astrocytes were then assessed by the percentage of the cells with GFAP fluorescence, accounting the whole number of the nucleus stained by Hoechst 33342 as described in Materials and Methods Section. A: The purities of astrocytes (%). Data are means ± SEM from five independent cultures of each procedure. $^{*}P < 0.05$, $^{**}P < 0.01$ versus primary culture; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus shaken once culture; $^{@}P < 0.05$ versus shaken twice culture. B: Representative photographs of anti-GFAP immunocytochemical staining and Hoechst 33342 staining. B-a: Anti-GFAP immunocytochemical staining of astrocytes prepared by SC. B-b: the image of B-a under light microscope; B-c: the overlapped image of both B-a and b; B-d: anti-GFAP immunocytochemical staining of astrocytes prepared by primary cultures; B-e: the corresponding Hoechst 33342 staining to B-d; B-f: anti-GFAP immunocytochemical staining of astrocytes prepared by shaken twice cultures; B-g: the corresponding Hoechst 33342 staining to B-f; B-h: anti-GFAP immunocytochemical staining of astrocytes prepared by shaken once cultures; B-i: the corresponding Hoechst 33342 staining to B-h; B-j: anti-GFAP immunocytochemical staining of astrocytes prepared by SC; B-k: the corresponding Hoechst 33342 staining to B-j. Arrows in B-d–k indicate the nucleus but their cell bodies are absent of GFAP immunocytochemical staining (scale bar = 50 μM). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

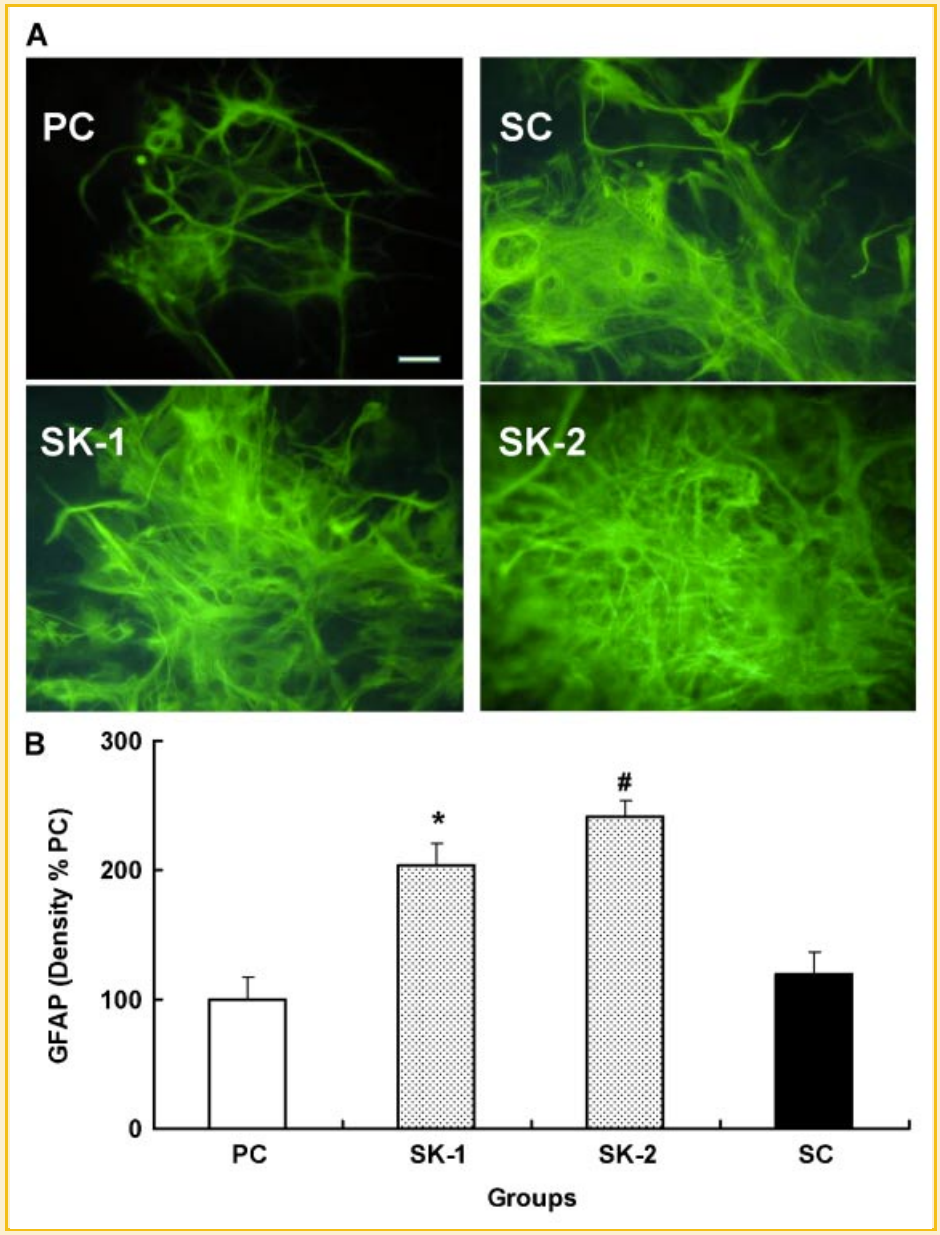

Fig. 2. Anti-GFAP immunocytochemical staining of astrocytes cultured by different procedures. Astrocytes were prepared from newborn ICR mice using different culture procedures and immunocytochemical staining were then performed as described in Materials and Methods Section. A: Representative photographs of anti-GFAP immunocytochemical staining (scale bar = 20 μM) (PC: primary culture; SC: subculture; SK-1: shaken once culture; and SK-2: shaken twice culture), (B) GFAP fluorescence density (% PC). Data are means ± SEM from three independent cultures of each procedure. $^{*}P < 0.05$ versus primary culture, $^{\#}P < 0.05$ versus primary culture and subculture. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

EFFECTS OF ISCHEMIA AND REPERFUSION ON THE VIABILITIES OF ASTROCYTES CULTURED BY DIFFERENT PROCEDURES

To find out whether there were any differences in the response of astrocytes cultured by different procedures to ischemia and reperfusion, we then investigated the effect of different durations of ischemia and reperfusion on the viabilities of astrocytes. Astrocytes in Hank's medium without glucose and serum (OGD) were exposed to 1% O_2 at 37°C for 0, 0.5, 3, 6, 9, 12, 18, 24, 36, or 48 h, and then to 21% O_2 (normoxia) for 24 h. Figure 4 showed the results of cellular viabilities. Although all response curves of cell viabilities to inschemia decreased progressively with the inschemic time, astrocytes cultured by shaken twice culture had a higher level of cell viabilities at most time points compared with astrocytes cultured by the three other procedures. At the time points of 12, 18, 24, 36, and 48 h, the cell viabilities of astrocytes cultured by shaken twice culture were significantly higher than those of the astrocytes cultured by PC, shaken once culture or/and SC ($P < 0.05$ or 0.01, Fig. 4). Data also showed that cell abilities in astrocytes obtained

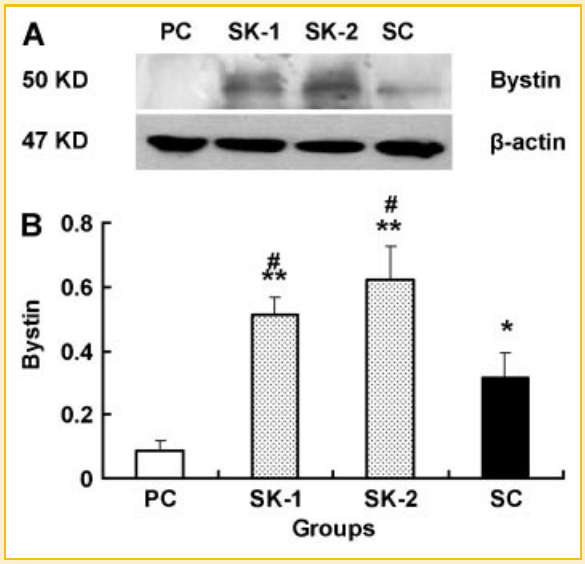

Fig. 3. Expression of bystin protein in astrocytes cultured by different procedures. Astrocytes were prepared from newborn ICR mice using different culture procedures (PC: primary culture; SK-1: shaken once culture; SK-2: shaken twice culture; and SC: subculture) and the expression of bystin protein was then examined by Western blotting analysis as described in Materials and Methods Section. A: Representative Western blot of bystin protein (a single band with a molecular weight of ~50 KD), (B) the relative values of bystin protein. Data are means ± SEM from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.001$ versus PC; $^{\#}P < 0.01$ versus SC.

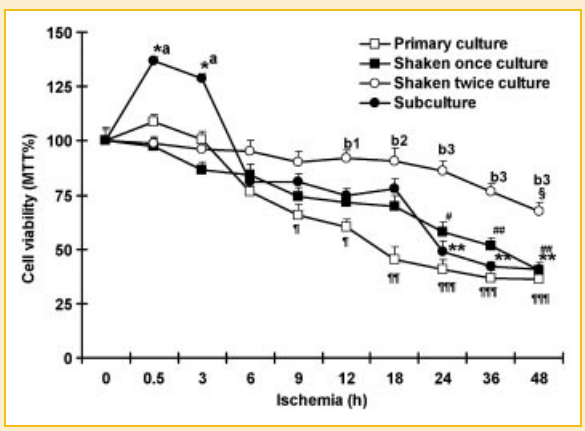

Fig. 4. Effects of ischemia and reperfusion on the viabilities of astrocytes cultured by different procedures. Astrocytes in Hank's medium without glucose and serum (oxygen–glucose deprivation) were exposed to 1% O_2 in an incubator (NAPCO 7101FC-1) with 94% N_2 and 5% CO_2 at 37°C for 0, 0.5, 3, 6, 9, 12, 18, 24, 36, or 48 h, and then exposed to 21% O_2 (normoxia) for 24 h. After the treatments, cellular viability was determined using MTT assay as described in Materials and Methods Section. Data are means ± SEM (n = 18). $^{¶}P < 0.05$, $^{¶¶}P < 0.01$, $^{¶¶¶}P < 0.001$ versus the control (PC: primary culture); $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus the control (SK-1: shaken once culture); $^{§}P < 0.05$ versus the control (SK-2: shaken twice culture); $^{*}P < 0.01$, $^{**}P < 0.001$ versus the control (SC: subculture). $^{a}P < 0.05$ or 0.01 versus PC, SK-1 and SK-2; $^{b1}P < 0.05$ versus PC; $^{b2}P < 0.05$ versus SK-1 and $P < 0.01$ versus PC; $^{b3}P < 0.05$ or 0.01 versus PC, SK-1, and SK-2.

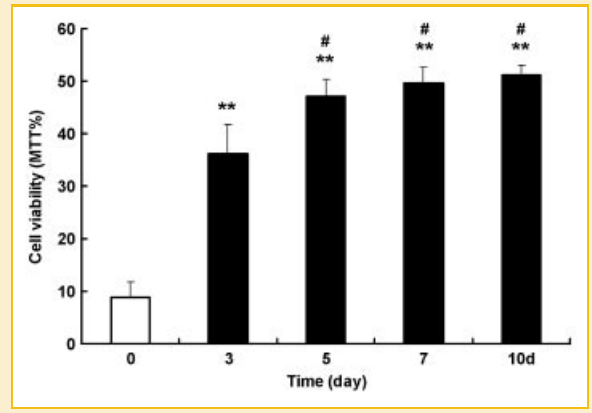

Fig. 5. Effects of ischemia and reperfusion on astrocytes which were further cultured for different days after the routine procedure of subculture. After the completion of the routine procedure of subculture (three times every 4 days only), astrocytes were further cultured for 0, 3, 5, 7, or 10 days and then exposed to ischemia (24 h) followed by reperfusion (24 h). After these treatments, cell viability was then determined by MTT assay. Results are means ± SEM (n = 18). $^{**}P < 0.001$, versus day 0; $^{\#}P < 0.05$, versus day 3.

from PC and SC were higher than those in the corresponding control at the time points of 0.5 and 3 h. However, statistical significance was only found in the subcultural group.

EFFECTS OF ISCHEMIA AND REPERFUSION ON ASTROCYTES FURTHER CULTURED FOR DIFFERENT DURATIONS AFTER THE ROUTINE PROCEDURE OF SUBCULTURE

In general, astrocytes were prepared by the routine procedure of SC (three times every 4 days only) and immediately used for experimental studies. In this case, the cells were not cultured for another 7 days after the last SC. Occasionally, the cells were not used immediately but a few days after the completion of the routine procedure of SC (RPS). It was unknown whether there were any differences in the characteristics of astrocytes that were used at different times after RPS. Therefore, we also investigated the effects of ischemia and reperfusion on the cell viability of astrocytes that were further cultured for different durations after the routine procedure of SC (RPS-d 0, 3, 5, 7, and 10). The astrocytes at RPS-d 0, 3, 5, 7, and 10 were exposed to ischemia (24 h) followed by reperfusion (24 h), and cell viability was then determined by MTT assay. The findings demonstrated the existence of significant differences in the responses of the cells to ischemia and reperfusion (Fig. 5). It was found that cell viabilities in astrocytes at RPS-d 3, 5, 7, 10 were significantly higher than those in astrocytes at RPS-d 0 ($P < 0.001$). The results also demonstrated that cell viabilities in astrocytes at RPS-d 3 were significantly lower than those in astrocytes at RPS-d 5, 7, 10 ($P < 0.05$). These suggest that astrocytes at RPS-d 0, 3, 5, 7, 10 have significant differences in their biological characteristics.

DISCUSSION

In the present study, we tested our hypothesis that different culture protocols might induce differences not only in the percentage of

astrocytes but also in their biological characteristics. Our findings provided clear evidence to support this hypothesis. We demonstrated that different culture procedures induced significant effect not only on the purity of astrocytes but also on their biological characteristics such as the percentage of activated astrocytes and GFAP and bystin expression. There was also evidence that differences in the biological characteristics of astrocytes could result in significant differences in the experimental results of the responses of cells to ischemia and reperfusion-induced injury.

The purity of astrocytes generated by the SC procedure was $98.9 \pm 1.081\%$. It was significantly higher than those by PC ($88.9 \pm 3.212\%$), shaken once culture ($94.8 \pm 1.325\%$) or shaken twice culture ($96.5 \pm 1.080\%$). In addition, expression of GFAP in astrocytes purified by the SC procedure (119.2% of PC) was at a significantly lower level as compared with those by shaken once culture (204.2% of PC) or shaken twice culture (242.2% of PC), with no significant differences found with that by PC. In the case of bystin, the content of this protein in astrocytes purified by the SC procedure was also significantly lower than those by shaken once culture or shaken twice culture, although there was a significant difference with that by PC. Because of the highest purity and the relatively lower expression of GFAP and bystin, the SC procedure could be considered as the optimal protocol to prepare astrocytes for in vitro study.

It was found that expression of GFAP and bystin in the astrocytes by the shaken once culture or shaken twice culture procedures is significantly higher than those in astrocytes prepared by PC or the SC procedures. GFAP is a major type of intermediate filament. As a member of the cytoskeletal protein family, it is believed that GFAP is important in modulating astrocytic motility and shape by providing structural stability to the astrocytic processes [Eng et al., 2000]. A significant increase in GFAP has been widely recognized as a marker of astrogliosis [Wu and Schwartz, 1998]. In addition to the increased GFAP with the activation of astrocytes, it has been recently reported that bystin, a protein potentially involved in embryo implantation [Suzuki et al., 1998, 1999], is markedly up-regulated in the reactive astrocytes of both 6-hydrodopamine-lesioned nigrostriatum and stab-lesioned cerebral cortex of adult rats in vivo and in postnatal cortical astrocytes treated with pro-inflammatory mediators lipopolysaccharide and interleukin-1b in vitro [Sheng et al., 2004]. It has also been suggested [Sheng et al., 2004] that bystin acts as a novel marker for reactive astrocytes in the adult rat brain following injury. Astrocytes become reactive (astrogliosis) in response to many pathologies of the CNS, such as stroke, trauma, growth of tumor, chemical insult, and neurodegenerative disease [Pekny and Nilsson, 2005; Sofroniew, 2005]. The significant increase in the expression of GFAP and bystin found in the astrocytes by the shaken culture procedures implies that physical stimulation induced by ''shaking'' might also able to activate astrocytes.

In consistent with the findings on GFAP and bystin expressions, astrocytes cultured by shaken twice culture and shaken once culture, but especially shaken twice culture, have a higher level of cell viabilities at most time points after ischemia compared with astrocytes cultured by PC or SC. The better tolerance of the cells to ischemia suggests that ''shaking'' might be capable of preconditioning as sublethal hypoxia [Murray et al., 1986] or ginkgolides [Zhu et al., 2008] to protect astrocytes against ischemic injury by the activation of astrocytes. On the other hand, astrocytes prepared by PC and the SC procedures have relatively low expressions of GFAP and bystin as compared with astrocytes cultured by the shaken culture procedures. This suggests that most of the cells in the cultures were still in an ''inactive state'' (quiescent astrocytes), hence the tolerance of these cells to ischemia was significantly lower than that of astrocytes cultured by shaken twice culture, as pointed by a lower level of cell viabilities at most time points after ischemia. It was also noted that there was a significant or slight increase in cell viabilities of astrocytes prepared by PC or the SC procedures at 0.5 and 3 h after ischemia. The increased cell viabilities at these two time points might be due to the increased number of activated astrocytes induced by ischemia [Du et al., 2008]. The purity of astrocytes generated by the SC procedure was significantly higher than that by PC. Hence, the number of the activated astrocytes in the SC group should be higher than that in the PC group. It is probably why cell viabilities in the SC group were significantly higher than the corresponding control, whereas those in the PC group were only slightly higher than the control at these two time points.

In addition to the various differences induced by the different culture procedures, we investigated the effects of ischemia and reperfusion on astrocytes which were further cultured for different durations after the routine procedure of SC. This investigation aimed to find out whether there was any significant effect of different culture periods on the biological characteristics of astrocytes. The measurements of cell viabilities demonstrated the existence of significant differences in the responses of the cells used at different days to ischemia and reperfusion, suggesting a significant difference in the biological characteristics of these astrocytes.

Based on our findings, it should be strongly emphasized that great attention must be paid to the significant differences in the biological characteristics of astrocytes induced by different culture procedures as well as different culture periods. These differences should not be underestimated because they could significantly affect experimental results as we reported in the present study. It is also necessary that journal authors, referees, and editors become aware of the effects of culture protocol factors on astrocytes in order to reduce the number of publications which interpret or compare data erroneously and incorrectly. It is also suggested that the methods of astrocyte culture including procedure and culture period used and other cultured conditions should be fully described in research papers. Our study is just a beginning to understand the effect of ''culture protocol factor'' on the biological characteristics of astrocytes. More detailed analysis on the fundamental issue of experimental methods of neurobiological study is absolutely needed.

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